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The analysis of diruthenium compounds on human cells as potential chemotherapeutic agents

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THE ANALYSIS OF DIRUTHENIUM COMPOUNDS ON HUMAN CELLS AS POTENTIAL CHEMOTHERAPUTIC AGENTS

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Abstract

Chemotherapy is a common method of cancer treatment, and cisplatin is a common anticancer compound used in chemotherapy. While effective, cisplatin is known to cause many side effects, and people can form resistance to it. Because of these problems, new chemotherapeutic compounds are needed. One compound that has shown anticancer properties, an ability to overcome resistance, and nontoxicity is ruthenium. Multiple mono-ruthenium compounds have been previously studied and were found to be effective. The current research project synthesized a diruthenium compound $Cp_2Ru_2(bipy)_2(DMSO)_2$ and exposed the compound to human liver cells (HepG2) to determine cytotoxicity. Increasing concentrations, between 5 and 50 μM, of the compound were tested, in addition to the solvent DMSO (control). Cytotoxicity effects were evaluated at 24, 48, and 72 hour time points. The results showed that $Cp_2Ru_2(bipy)_2(DMSO)_2$ produced significant decreases in cell growth at 10 μM concentrations and was more effective at the 24 hour time point when compared to cisplatin, however, 15 μM cisplatin was more effective at 48 and 72 hour time points. Additionally, there were solvent effects with DMSO at higher concentrations of the diruthenium compound (25 and 50 μM). At low concentrations, solvent did not show an effect.

Introduction

Cancer is a disease that will affect almost 40% of people in their lifetime. There are over 100 types of cancer, with the most common types being breast, prostate, lung, and colon (National Cancer Institute, 2015). As of 2015, there are 454.8/100,000 new cases of cancer with 171.2/100,000 deaths from cancer each year. There are over 13.7 million people in the United States living with cancer (National Cancer Institute, 2015). Because of the prevalence of cancer, finding new treatments has been an important goal of many researchers. One common method of cancer treatment is chemotherapy, which is the use of medications to destroy cancer cells in the body. Many of these compounds target cancer cells but can also affect nearby normal cells. Thus, chemotherapy can cause many side effects and decrease a person's quality of life due to the many side effects. People can also develop resistance to chemotherapy, making the treatment not effective for them. Because of these concerns, safer and more effective forms of chemotherapy need to be discovered.

Ruthenium has been shown to have anticancer properties, nontoxic, and able to overcome resistance (Bergamo et al., 2012). We wanted to synthesize a compound with a two ruthenium centers and compare it to cisplatin, a popular chemotherapeutic drug. The goal of this research is to determine how well this novel diruthenium compound is able to decrease cell growth to compare to cisplatin.

Literature Review

Cancer

Cancer is characterized by uncontrolled cell division that spreads into surrounding tissues. In a healthy individual, cells divide, form new cells, and die when appropriate. In a patient with cancer, cells may not die when they are supposed to and new cells grow when not needed (National Cancer Institute, 2015). Cells that divide without stopping can form tumors, and cancerous tumors that form are called malignant tumors.

Cancer is caused by genetic changes in an individual. Mutations occur in the DNA of cancer patients, and the changes may be a cause or a result of the cancer. Genetic changes can be inherited from parents or caused by DNA damage from environmental factors including smoking and ultraviolet radiation (Greenblatt et al., 1994). There are many types of cancer that are characterized by where they are located in the body.

Chemotherapy

Chemotherapy is a common method of treatment for cancer. Chemotherapy uses pharmaceutical medications to destroy cancer cells in the human body. One group of medications used in chemotherapy can target cell division (Payne and Miles, 2008). Cancer cells are known to divide more than normal cells, but chemotherapy is not specific to cancer cells and can target any dividing cell in the body. Since chemotherapy can target any dividing cell, it affects other rapidly dividing cells including hair, nails, and bone marrow (Payne and Miles, 2008).

Most types of chemotherapy damage the genetic material in a cell to cause cell death (Payne and Miles, 2008). There are four types of cellular activities that cause cell death: apoptosis, necrosis, autophagy, and mitotic catastrophe (Ricci and Zong, 2006). Apoptosis and autophagy are types of programmed cell death, which means there is genetic control over the

processes. Programmed cell death involves absorption of cellular components by nearby cells and is used by healthy cells to maintain normal tissues (Ricci and Zong, 2006). Necrosis and mitotic catastrophe are typically thought of as accidental cell death which are caused by either physical damage to the cell or abnormal cellular division. These are characterized by dysregulation of the signaling pathways that control cell death. The various types of chemotherapy have been shown to induce all types of cell death (Ricci and Zong, 2006).

Although it can be an effective method of cancer treatment, chemotherapy has downsides. It can damage DNA in normal cells leading to cell death, causing many side-effects Payne and Miles, 2008). Side effects can include but are not limited to nausea, vomiting, hair loss, constipation, shaking, and diarrhea (Boer-Dennert et al., 1997). Also, some people are resistant to forms of chemotherapy. For some, chemotherapy does not improve the status of the cancer and may not be effective.

Cisplatin

Cisplatin, developed in 1965 and approved by the Food and Drug Administration (FDA) in 1978, is classified as an alkylating agent and used for a variety of cancers including testicular, ovarian, bladder, esophageal, breast, cervical, stomach and prostate.

Side effects of cisplatin occur in greater than 30% of the patients receiving the medication. These include nausea, vomiting, kidney toxicity, blood test abnormalities, low white blood cell count, and low red blood cell count. Women who are pregnant are informed of the potential risks of cisplatin on a developing fetus. Cisplatin is a pregnancy category D drug, which means there is risk of harm to an unborn baby but the risks may outweigh the benefits for the mother (U.S. Department of Health and Human Services, 2015). It is recommended for

patients who are undergoing chemotherapy with cisplatin to take anti-nausea medications, eat small amounts of food regularly, maintain regular fluid intake, wash hands often, avoid sun exposure, and get plenty of rest.

Since cisplatin is classified as an alkylating agent, it is most active in the resting phase of the cell and is cell cycle non-specific (Larsen, 2013). An alkylating agent is a type of anticancer drug that inhibits transcription of DNA into RNA by substituting alkyl groups for hydrogen on DNA, which causes crosslinking in the DNA (National Library of Medicine, 2015). This results in mutated DNA. Cisplatin is neutral in charge upon entering the cell (Larsen, 2013). Once inside a cell, cisplatin undergoes hydrolysis, where a chlorine ligand is replaced by a molecule of water (Larsen, 2013). This creates a positively charged species, as shown in **Figure 1**.

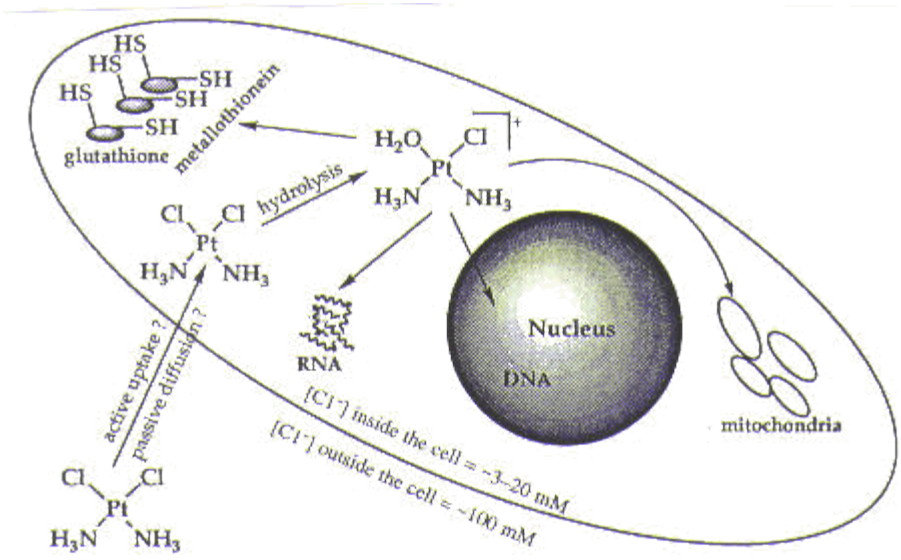

Figure 1. Cellular uptake of cisplatin (Larsen, 2013)

Once inside the cell, cisplatin has a number of possible targets including DNA, RNA, sulfur-containing enzymes such as metallothionein and glutathione, and mitochondria.

Metallothionein and glutathione have been shown to increase cellular resistance to cisplatin, making cisplatin less effective (Basu and Krishnamurthy, 2010). The effects on mitochondrial DNA are not well understood, but it is possible that damage to mitochondrial DNA resulting from cisplatin treatment contributes to cell death (Larsen, 2013).

A downside to cisplatin use is the resistance that can occur. Once cisplatin interferes with DNA replication, it activates the cell's repair mechanisms and undergoes cell death if unable to repair (Oliver et al., 2010). It has been shown that cells treated with cisplatin alter the DNA repair pathways, allowing them to become resistant to cisplatin (Oliver et al., 2010). Research shows that tumor suppressor gene p53 can become mutated, resulting in a higher chance of resistance to cisplatin. Another downside of cisplatin is the many side effects that can occur, as previously described. Because of these issues with cisplatin, other drugs are being developed and tested for anticancer abilities, including ruthenium compounds.

Ruthenium

Ruthenium, atomic number of 44, was discovered in 1840 by Karl Karlovich Klaus after he extracted, purified, and confirmed the new metal (RSC, 2015). Ruthenium has many uses in the electrical and chemical industries and is an alloying agent when combined with metals such as platinum and palladium (Seddon and Seddon, 1984). Ruthenium is considered highly toxic and carcinogenic.

Work with ruthenium as an anticancer drug began between 1975 and 1985 and lead to a few important findings that include: the hypothesis of "activation by reduction", the transportation of ruthenium to cancer cells by transferrin, and DNA binding different from platinum drugs (Bergamo and Sava, 2011). Ruthenium is of interest to researchers because it is

believed to be less toxic and more capable of overcoming resistance when compared to cisplatin (Bergamo and Sava, 2011). This seems to occur by the transportation of ruthenium to the tumor cells by transferrin and the selective activation to more reactive species by the reducing environment of tumors when compared to healthy tissues (Bergamo and Sava, 2011). Ruthenium anti-cancer medications are designed to mimic cisplatin, particularly for targeting DNA, although they may alter DNA differently (Bergamo and Sava, 2011).

While there are similarities, ruthenium-based complexes have been shown to act differently from cisplatin in that ruthenium accumulates in neoplastic masses rather than healthy tissue, and then can lead to cellular apoptosis (Antonarakis and Emadi, 2010). Additionally, ruthenium remains inactive until it reaches the tumor. With many tumors being resistant to cisplatin, this allows ruthenium to perhaps attack tumors that would normally be more resistant to chemotherapy and radiation (Antonarakis and Emadi, 2010). Ruthenium shows an ability to fight metastasis in addition to the primary tumor by interfering with type IV collagenolytic activity to reduce metastasis (Antonarakis and Emadi, 2010).

Research has been conducted with mono-ruthenium compounds as chemotherapeutic and diagnostic agents. Previous research has shown that ruthenium can be used as an anti-inflammatory agent, antibiotic, antidiabetic agent, antioxidant, and anti-analgesic agent among other uses (Naik et al., 2015). Two mono-ruthenium compounds have been clinically tested, NAMI-A and KP1019. KP1019 mimicked cisplatin and had potential to overcome resistance and possibly systemic toxicity because of its different mode of action on the nucleic acid (Bergamo et al., 2012). KP1019 also showed some cancer stabilization in patients and a phase II study with colorectal cancer patients is being planned (Antonarakis and Emadi, 2010). NAMI-A showed an ability to act on tumor metastasis (Bergamo et al., 2012). NAMI-A appears to

interfere with type IV collagenolytic activity to reduce the metastatic potential, instead of binding to DNA (Naik et al., 2015). The structures of the two compounds are shown below in **Figure 2**.

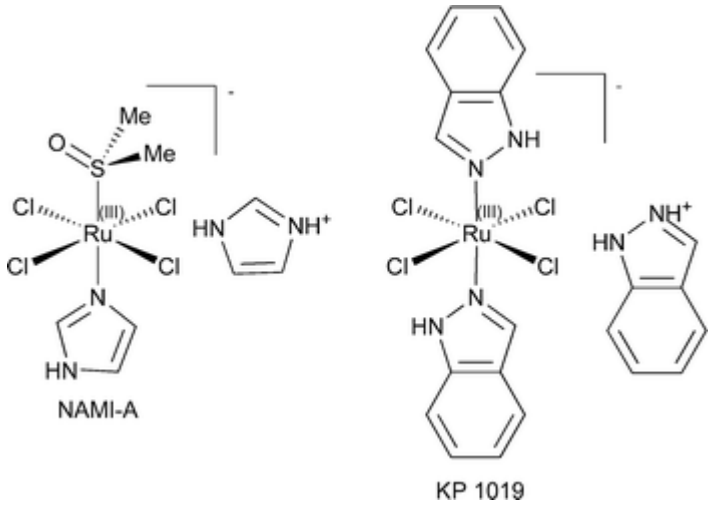

Figure 2. Structures of NAMI-A and KP2019 (Gianferrara et al., 2009)

Current research project

The current research project seeks to evaluate if a diruthenium compound is cytotoxic to human liver cells (HepG2). Since mono-ruthenium compounds have been shown to have anti-cancer properties, the goal was to analyze how effective a diruthenium compound that was synthesized in the laboratory was in producing cell death. First, a diruthenium compound ($Cp_2Ru_2(bipy)_2(DMSO)_2$) was synthesized and exposed to target cells. Our goal was to evaluate the toxicity of the compound on target cells and to compare those results with cisplatin, a known and effective chemotherapeutic drug. (**Figure 3**).

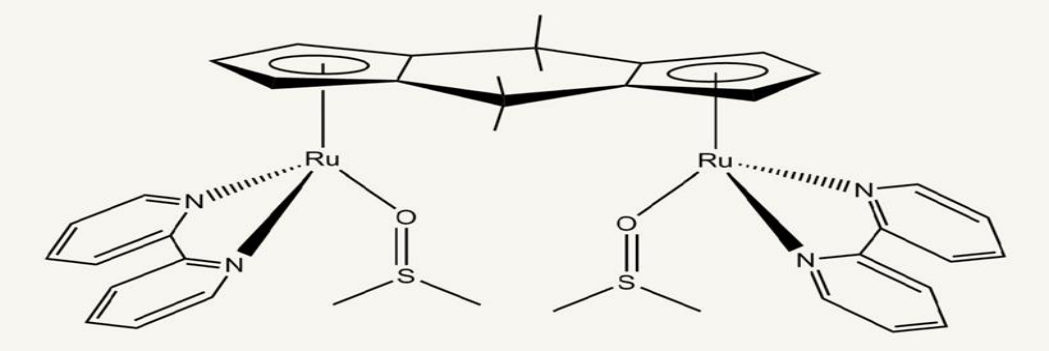

Figure 3. Structure of $Cp_2Ru_2(bipy)_2(DMSO)_2$

Our hypothesis was that the diruthenium compound would be more toxic than cisplatin. The goal of the project was to provide insight into the potential chemotherapeutic activity that diruthenium compounds can have as anti-cancer agents. We determined the concentrations at which these compounds can produce at least 50% cytotoxicity, and determined their effectiveness compared to the very commonly used drug cisplatin. The hope is that the current project can lead to further research with diruthenium or other ruthenium compounds.

Methods

Chemical synthesis of Ruthenium compound

Silver triflate, naphthalene, and the starting material $Cp_2Ru_2(CO)_4(Br)_2$ were used to synthesize a new compound in the nitrogen glove box. Acetonitrile was added to solubilize the materials, and the solution was microwaved for 20 minutes at 180^o C. After microwaving, the mixture was transferred into diethyl ether where the insoluble material was separated by filtration. The filtration was followed by a methylene chloride wash. The compound was then

dried, rinsed with tetrahydrofuran (THF), rinsed with diethyl ether once again, and vacuum dried at room temperature.

The next day, acetonitrile was added to the compound, (Cp_2Ru_2Np), and the solution was photolyzed (visible light) for 3 hours. NMR spectral analysis was performed following the 3 hour period to determine if the reaction was complete. Half of the compound (yellow liquid) was evaporated by the vacuum and bipyridine was added. The solution was microwaved for 20 minutes at 80^o C. The solution changed from a yellow color to dark red. Lithium chloride (LiCl) was added to the dark red mixture, and the mixture was microwaved again for 20 minutes at 80^o C. The mixture was left stirring on a stir plate overnight.

Finally, the compound $[Cp_2Ru_2(bipy)_2(Cl)][OTf]$ was evaporated to produce a dark powder (bipy is 2,2'-Bipyridine). The compound was dissolved in DMSO, which released the chloro ligand thereby producing $Cp_2Ru_2(bipy)_2(DMSO)_2$.

$Cp_2Ru_2(bipy)_2(DMSO)_2$ Solution

3.5 mg of $Cp_2Ru_2(bipy)_2(DMSO)_2$was dissolved in 1 mL of DMSO to create a working test solution that was used to treat cells.

Cell culture

The HepG2 cell line, a non-tumorigenic human hepatocellular carcinoma, was used. The cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2. They were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum

(FBS), and 1% penicillin/streptomycin. Cells were subdivided (split) when the dish was 80-85% confluent or full using trypsin digestion.

Cell Treatment for MTT proliferation assay

On day 1, HepG2 cells were plated in medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. For each experiment, 10,000 cells/well were plated in 96-well plates.

On the 2nd day, old medium was removed and replaced with medium containing increasing concentrations of $Cp_2Ru_2(bipy)_2(DMSO)_2$. The concentrations ranged from 5-100 μM. DMSO solvent controls were used as comparison for each concentration. The positive control was 15 μM Cisplatin and the same volume of NaCl was also used as the solvent control comparison. On days 3, 4, and 5, 24, 48, and 72 hour MTT cell proliferation assays were performed.

MTT Cell Proliferation Assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a colorimetric assay that assesses cell viability. MTT is a yellow colored compound but turns into a purple formazan salt when reduced by mitochondrial enzymes of viable cells only. A MTT assay kit (ATCC) was used initially. Ten μL of 5 mg/mL MTT reagent was added to each well, followed by the plate being incubated for 2 hours until purple formazan appeared. One hundred μL of SDS stock solution (10% Sodium Dodecyl Sulfate in 0.01 N HCL) was added per well and the plate was incubated in the dark for an additional two hours. Absorbance was measured at 570 nm on a spectrophotometer. After 4 weeks, MTT compound (Sigma) was purchased separately and dissolved in phosphate buffered saline (5 mg/mL). The same stop solution was used. For the MTT assay, 20 μL of MTT solution (5 mg/mL) was added to each well and plates were

incubated for 4 hours at 37° C. One hundred µL of stop solution was added and the plate was incubated overnight at 37° C with plates being read (570 nm) on the spectrophotometer for determination of the absorbance of each sample the following morning.

Data Analysis

Data analysis using statistical software (SPSS) to determine the average amount of decrease in cell proliferation for each concentration at each time point was conducted. Three 24 hour experiments, four 48 hour, and three 72 hour experiments were analyzed with 8 replicates for each concentration. One Way ANOVA with Bonferroni post-hoc analysis was used to determine statistical significance where $p<0.05$ was considered statistically significant. To compare how treated cells behaved compared to control (untreated cells) a ratio of the absorbance of treated cells divided by the absorbance of the control cells was calculated.

Results

Synthesis of $Cp_2Ru_2(bipy)_2(DMSO)_2$

After the synthesis of the compound shown in Figure 3, it was assumed that the chlorine ligand became unbound from the ruthenium metal centers and that DMSO was bound to the metal centers instead. The compound was used for comparison against cisplatin in MTT cell proliferation assays.

MTT Cell Proliferation Analysis

Following MTT cell proliferation assays, the concentrations of 5, 10, 25, and 50 µM of $Cp_2Ru_2(bipy)_2(DMSO)_2$ were compared to DMSO solvent control counterparts. Cisplatin (15 µM) was used as the positive control and its solvent NaCl was also included. Figure 4 shows

the results following 24 hours of exposure to HepG2 cells. The 10 µM concentration decreased cell growth by 15%, 25 µM by 22%, and 50 µM by 24%; all of these concentrations showed a statistically significant decrease in cell growth compared to control. Concentrations of DMSO did not decrease cell growth while the cisplatin control decreased cell growth by 2% NaCl did not.

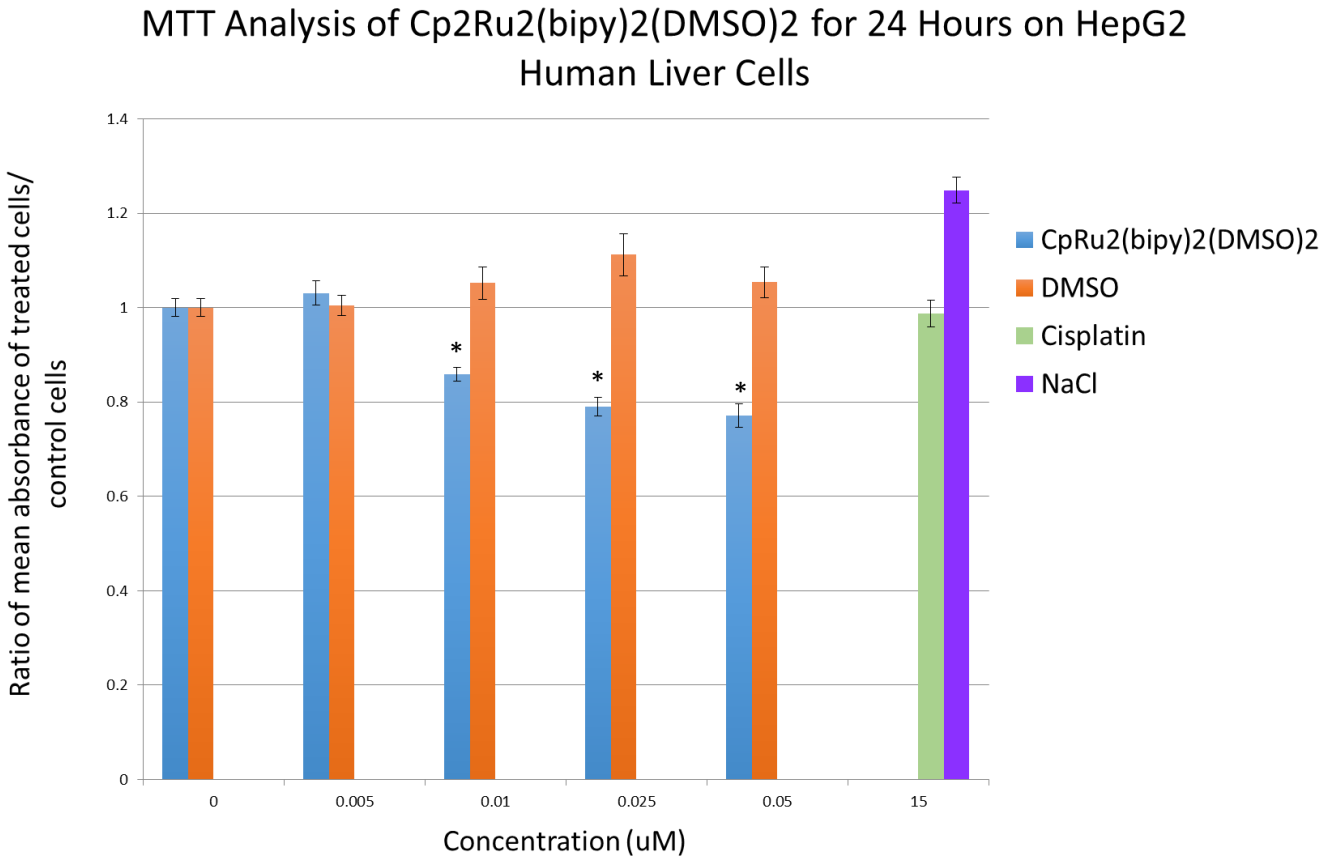

Figure 4. At 24 hours, 10 µM $Cp_2Ru_2(bipy)_2(DMSO)_2$ on HepG2 cells showed 15% decrease in cell growth. $Cp_2Ru_2(bipy)_2(DMSO)_2$ showed a statistically significant change (*) from control with $p < 0.05$ at 10, 25, and 50 µM.

Following a 48 hour exposure of $Cp_2Ru_2(bipy)_2(DMSO)_2$ to HepG2 cells, cell growth was assessed by comparing increasing concentrations of compound to similar volumes of the DMSO solvent control, and the positive control. Five µM of the compound decreased cell growth by 15%, 10 µM decreased growth by 19%, 25 µM decreased growth by 37%, while 50 µM

decreased cell growth by 50%. $Cp_2Ru_2(bipy)_2(DMSO)_2$ after a 48 hour exposure showed a significant change from control with all the concentrations tested. However, DMSO alone also 25%. Cisplatin also showed a significant change from control, decreasing cell growth 37% while its solvent control did not (Figure 5).

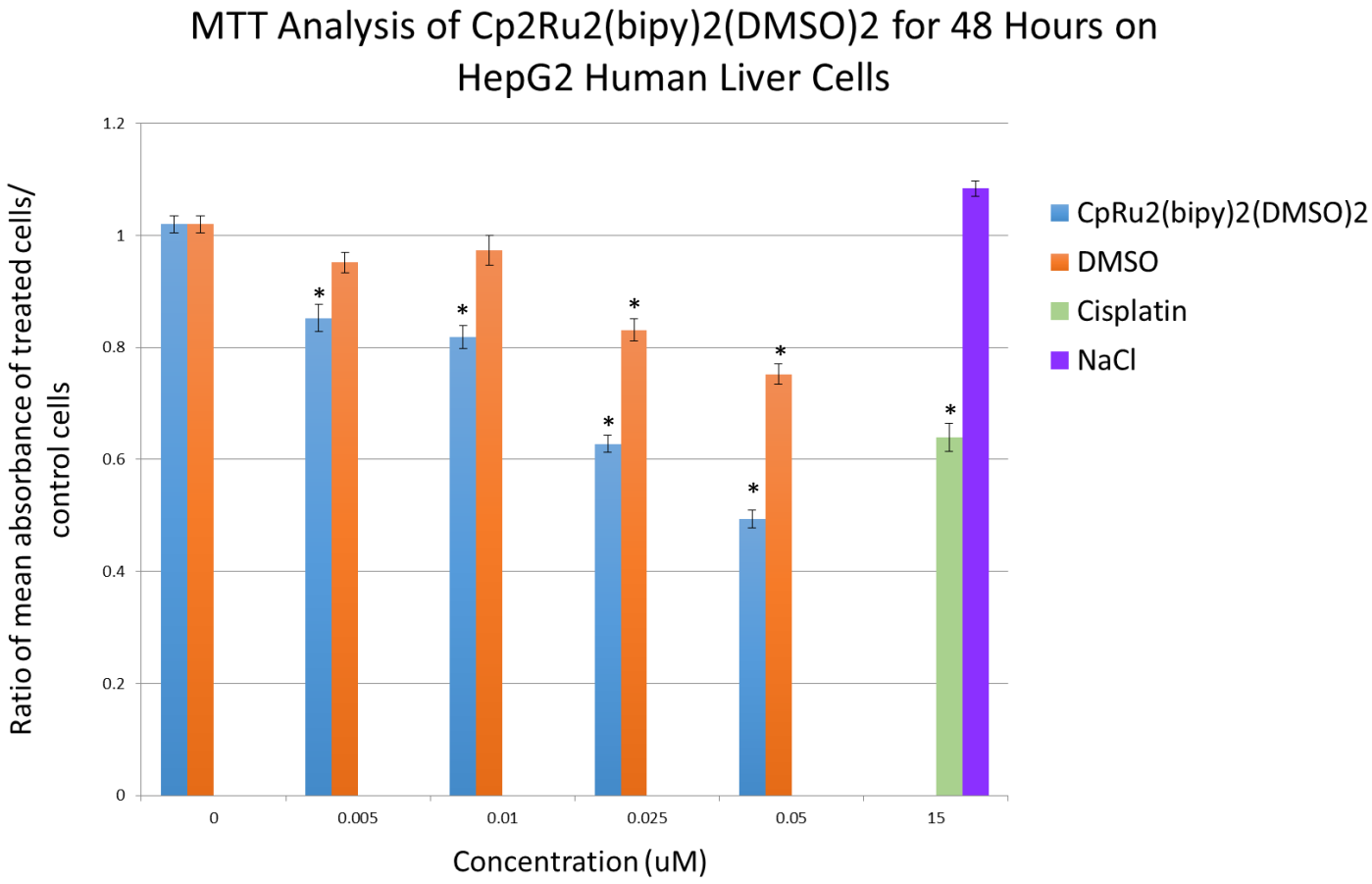

Figure 5. At 48 hours, 5 μM $Cp_2Ru_2(bipy)_2(DMSO)_2$ on HepG2 cells showed 15% decrease in cell growth. $Cp_2Ru_2(bipy)_2(DMSO)_2$ showed a significant change (*) from control with $p < 0.05$ at 5, 10, 25, and 50 μM. DMSO showed a significant change from control with $p < 0.05$ at 25 and 50 μM. Cisplatin also showed a significant change from control.

Following exposure of $Cp_2Ru_2(bipy)_2(DMSO)_2$ for 72 hours to HepG2 cells, cell growth was assessed by comparing increasing concentrations of compound to similar volumes of the

DMSO solvent control, and the positive control. So, after 72 hour exposure, the concentration of 5 µM deceased cell growth by 10%, 10 µM decreased growth by 15%, 25 µM decreased growth by 36%, and 50 µM decreased growth by 58%; all the concentrations were statistically significant in decreasing cell growth compared to control cells. However, DMSO showed a significant change from control with $p < 0.05$ at the higher concentrations, 25 and 50 µM, decreasing cell growth by 22% and 43%. Cisplatin also showed a significant change from control, decreasing cell growth 88% while the solvent NaCl was not significant from control.

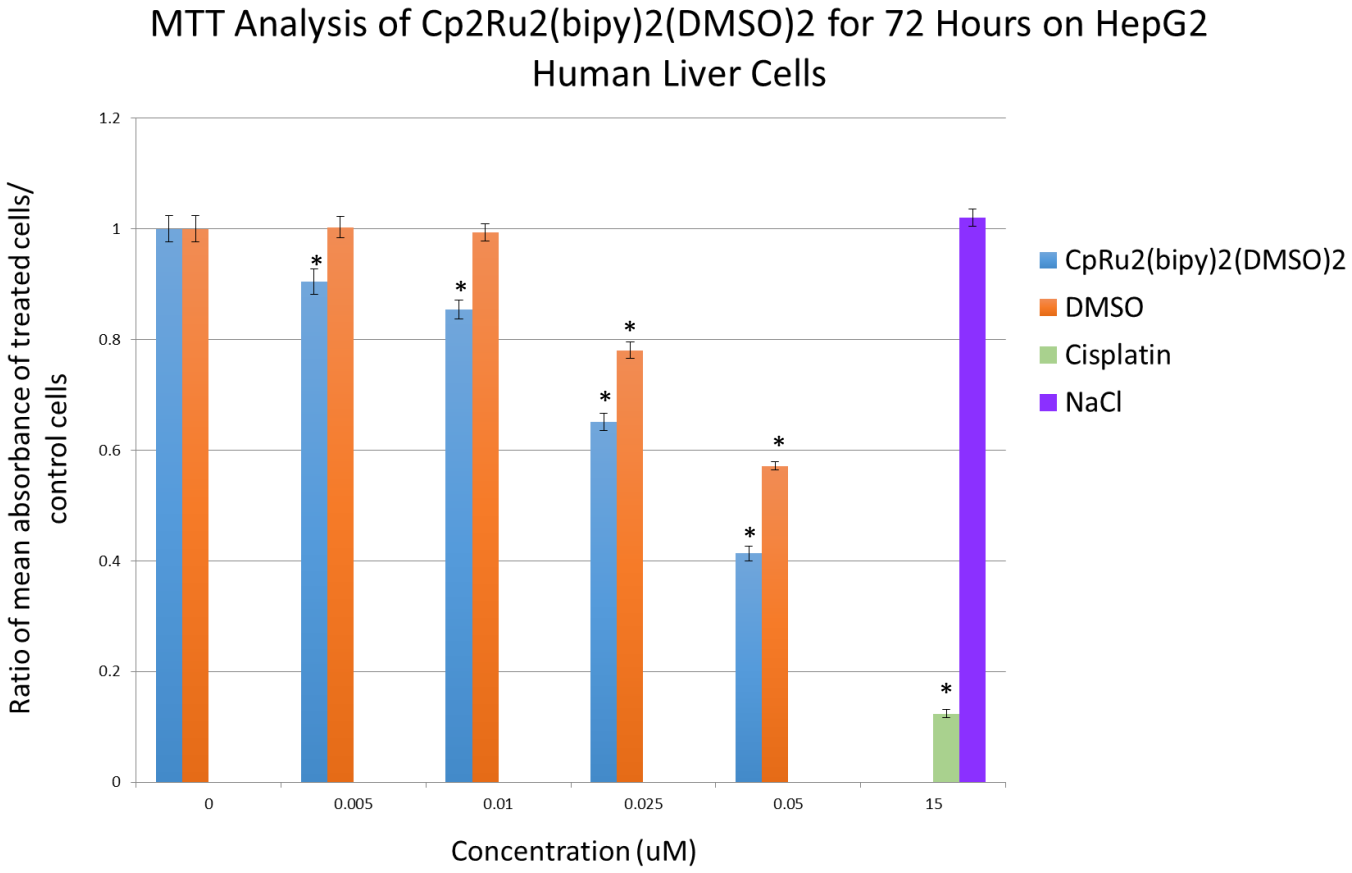

Figure 6. At 72 hours, 5 uM $Cp_2Ru_2(bipy)_2(DMSO)_2$ on HepG2 cells showed 10% decrease in cell growth. $Cp_2Ru_2(bipy)_2(DMSO)_2$ showed a significant change (*) from control with $p < 0.05$ at 5, 10, 25, and 50 µM. DMSO showed a significant change from control with $p < 0.05$ at 25 and 50 µM. Cisplatin also showed a significant change from control.

Discussion

In the search for new compounds with anticancer properties, diruthenium compound $Cp_2Ru_2(bipy)_2(DMSO)_2$ was synthesized and tested against a known positive control, cisplatin, a common compound used in chemotherapy. Cisplatin can cause many side effects in patients, and patients can develop primary or secondary resistance to the compound, creating a need for new anticancer compounds. We wanted to know how $Cp_2Ru_2(bipy)_2(DMSO)_2$ compared to cisplatin in terms of cytotoxicity.

The results from the MTT analysis of HepG2 cells showed that only 10 μM of $Cp_2Ru_2(bipy)_2(DMSO)_2$ was needed to produce a significant decrease in cell growth after 24 hours (Figure 4). However, only 5 μM of compound was needed to produce a significant decrease in cell growth after 48 and 72 hours, as shown in Figure 5 and Figure 6. At these concentrations, there was no solvent effect seen with DMSO alone.

Cisplatin (15 μM) was used as a positive control for all time points. It did not significantly affect cell growth at 24 hours, but was significant at both 48 and 72 hours. Cisplatin showed the greatest decrease in cell growth at the 72 hour time point by decreasing cell growth by 85% while the diruthenium compound decreased growth by 60% at 50 μM after 72 hours, however, for the compound, there was a solvent effect at this concentration, thus we cannot assume the effect was due only to the compound but it may also be due to the solvent. At lower concentrations of compound, there was no such solvent effect; it was only seen at higher concentrations at longer time points. These results indicate that $Cp_2Ru_2(bipy)_2(DMSO)_2$ can produce significant decreases in cell growth at low concentrations and that it was more effective at the 24 hour time point when compared to cisplatin, however, 15 μM cisplatin is more effective at longer time points. It is unknown if the diruthenium compound achieved greater cell death

than a mono-ruthenium compound because there was not a mono-ruthenium compound tested to compare to.

Future steps of this research could include determining the mechanism of cell death the cells undergo when exposed to $Cp_2Ru_2(bipy)_2(DMSO)_2$, whether it be apoptosis or necrosis. It could also include developing alternate synthesis methodologies that would allow the solubility of diruthenium compounds in aqueous solvents. Finally, $Cp_2Ru_2(bipy)_2(DMSO)_2$ could be evaluated for potential toxicity in tumorigenic cell lines.

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